RyR1 and its protein ligands are often not stable in vitro and therefore difficult to study by single-particle cryo-electron microscopy (cryo-EM). The main goal of this study was to develop a procedure to cross-link protein ligands to RyR1 and visualize these complexes by single-particle cryo-EM. To test the cross-linking protocol we used the complex of FK506-binding protein and RyR1 (FKBP12:RyR1) as a model system, since the structure of FKBP12:RyR1 is known. Glutaraldehyde quantitatively cross-linked RyR1 subunits to each other and FKBP12 to RyR1 without damaging the ultrastructure. Cross-linked FKBP12:RyR1 was visualized in 2D averages, and was identical to that of previously published non-cross-linked FKBP12:RyR1. The effect of glutaraldehyde cross-linking on RyR1 structure was characterized using 3D single-particle cryo-EM and by [3H]ryanodine binding assay. Glutaraldehyde cross-linking preserved the gross morphology of RyR1, but induced minor structural changes at the cytoplasmic and transmembrane regions of RyR1. Glutaraldehyde cross-linking enhanced [3H]ryanodine binding to RyR1 by ~30%. Based on these results we propose that cross-linking RyR1 subunits by glutaraldehyde locked RyR1 in an open-like conformation state.

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FRET-based Structural Measurements of the Type 1 Ryanodine Receptor using Site-Specific Fluorophore Labeling to Tetracysteine Motifs

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The type 1 ryanodine receptor (RyR1) is an intracellular Ca^{2+} release channel that mediates skeletal muscle excitation contraction coupling. While the overall shape of RyR1 has been elucidated using cryo electron microscopic reconstructions, fine structural details remain elusive. To better understand the structure of RyR1, we have previously used a fluorescence resonance energy transfer (FRET)-based method using a fused green fluorescent protein (GFP) donor and a fluorescent acceptor, Cy3NTA that binds specifically to short poly-histidine 'tags' engineered into RyR1. However, the large size of the GFP fusions and the need to permeabilize cells expressing these constructs (to allow Cy3NTA entry) limits interpretation of the resulting FRET data. To overcome these problems, we used a dodecapeptide sequence containing a tetracysteine (Tc) motif to target the bi-arsenical fluorophores, FlAsH and ReAsH to RyR1. These compounds freely cross intact cell membranes where they then bind covalently to the tetracysteine motif. First, we used this system to conduct FRET measurements in intact cells by fusing a YFP FRET donor to the N-terminus of RyR1 and then targeting the FRET acceptor, ReAsH to an adjacent Tc tag. High levels of energy transfer (~50%) were observed whereas incubation of ReAsH with a YFP-RyR1 fusion protein lacking the Tc tag resulted in no detectable FRET. We also developed a FRET-based system that did not require GFP fusions into RyR1 by labeling N-terminal Tc-tagged RyR1 with FlAsH, a FRET donor and then targeting the FRET acceptor Cy3NTA to an adjacent His tag. A high degree of energy transfer (~70%) indicated proper binding of both compounds to these unique recognition sequences in RyR1. Thus, these two systems provide unprecedented flexibility in FRET-based structural determinations of RyR1. Supported by NIH grant R01AR059124.

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Structural Mapping of Divergent Region Domains in the Type 1 Ryanodine Receptor using Two Complementary FRET-based Approaches

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We used fluorescence resonance energy transfer (FRET) to localize three divergent region domains within the type 1 ryanodine receptor (RyR1), an intracellular Ca^{2+} channel that mediates skeletal muscle excitation-contraction (EC) coupling. Initial cloning studies of the three RyR isoforms identified three ''divergent regions'' of primary sequence dissimilarity spanning amino acids 4254-4631 (DR1), 1342-1403 (DR2) and 1872-1923 (DR3) in RyR1. These regions have been implicated in EC coupling as well as in differential sensitivity to pharmacological agonists. Here, we used permeabilized HEK-293T cells expressing recombinant RyR1 to localize these DRs to the cryo electron microscopic (EM) map of RyR1. First, we measured FRET from a green fluorescent protein (GFP) donor fused to either position 1 or 620 of RyR1, to a FRET acceptor, Cy3NTA, targeted to poly-histidine ''tags'' inserted into DR1 (at position 4429), DR2 (at position1358) or DR3 (at position 1915). While FRET was not detected for His-tagged constructs containing GFP fused at position 1, FRET was observed from GFP fused at position 620 to all 3 His-tagged positions. Second, we targeted a donor to the RyR1 cytoplasmic domain using FKBP12.6 labeled with Alexa Fluor 488, and then measured FRET to Cy3NTA targeted to the His tag sites described above. Donor-FKBPs bound with high-affinity to both recombinant wild type and His-tagged RyRs. FRET was detected from donor conjugated to each of four, well-separated positions on FKBP to Cy3NTA targeted to each divergent region. Since the fused GFPs and FKBP12.6 have already been localized within the cryo EM map of RyR1, we can now triangulate the DR positions to the cryo EM map from these two complementary data sets. Supported by NIH grant R01AR059124 (to JDF, MM, and TG) and R01HL092097 (to RLC).

2281-Pos Board B300

The General Anaesthetic Binding Site of Calmodulin Disrupts Ryanodine Peptide Binding

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The skeletal muscle Ryanodine Receptor (RyR1) is a large calcium release channel involved in excitation-contraction coupling. It is also the target for hundreds of disease mutations that cause malignant hyperthermia (MH) or skeletal muscle disorders like central core disease (CCD). MH is typically triggered by volatile anesthetics, but their binding site on RyR1 has not been fully described. RyR1 is under the control of several auxiliary proteins. One of these is Calmodulin (CaM), a Ca^{2+}-binding protein that can suppress RyR1 activity at elevated Ca^{2+} concentrations. Here we investigate how CaM can bind to RyR1, and how this may be affected by volatile anesthetics. We found that CaM can bind to at least three different RyR1 peptides, with the affinity and lobe specificity being altered substantially by the Ca^{2+} concentrations. In addition, we identified two binding sites for sevoflurane, a volatile anesthetic, on Ca^{2+}/CaM. The anesthetic binds to a pocket that is involved in binding RyR1 peptides. In addition, it can alter the affinity of the N-terminal CaM lobe for Ca^{2+}. These findings suggest that binding of anesthetics to CaM may be involved in the pathophysiology of malignant hyperthermia.

2282-Pos Board B301

The Cytoplasmic Foot of RyR1 without the Membrane Spanning Domain Targets Junctionally and Retrogradely Enhances DHPR L-Type Ca^{2+} Currents

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In skeletal muscle, RyR1 (5,037 residues) forms a homo-tetrameric Ca^{2+}-release channel in the sarcoplasmic reticulum (SR), mediates excitation-contraction coupling in response to an orthograde signal from the DHPR in the plasma membrane, and retrogradely enhances L-type Ca^{2+} current via the DHPR. The RyR1 C-terminus forms the Ca^{2+} channel pore across the SR membrane and is believed to be important for inter-subunit interactions, whereas the bulk (~85%) of the protein (the so-called foot) bridges the junctional, myoplasmic gap between the SR and plasma membranes. Here, we have examined the ability of the foot domain (residues 1-4300; $RyR1_{1:4300}$) to target junctionally and interact with the DHPR by expression of a cDNA encoding YFP-$RyR1_{1:4300}$. In dysgenic (α_{1S}-null) myotubes which lack DHPRs, YFP-$RyR1_{1:4300}$ was diffusely distributed and, on the basis of photobleaching, freely mobile within the cytoplasm, consistent with the loss of membrane anchoring. However, after expression in dyspedic (RyR1 null) myotubes (which have DHPRs), much of YFP-$RyR1_{1:4300}$ was immobile within fluorescent foci near the myotube surface, suggestive of junctional targeting and binding to DHPRs. Junctional targeting was confirmed by partial co-localization of YFP-$RyR1_{1:4300}$ and CFP-labeled α_{1S} after co-expression in dyspedic myotubes. Strikingly, YFP-$RyR1_{1:4300}$ was able to retrogradely enhance peak Ca^{2+} current in dyspedic myotubes from 1.6 pA/pF (control) to 6.7 pA/pF, similar to that after expression of full-length RyR1 (7.4 pA/pF). Thus, the isolated, cytoplasmic foot of RyR1 retains the ability to target junctionally and to interact functionally with the DHPR.

2283-Pos Board B302

The Cytoplasmic Foot of RyR1 forms a Stable Homotetrameric Structure Despite Lacking the Membrane-Spanning C-Terminal Domains

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In skeletal muscle, the dihydropyridine receptor (DHPR) in the plasma membrane engages in bi-directional interactions with the type 1 ryanodine receptor (RyR1) in the sarcoplasmic reticulum (SR) such that an ''orthograde'' signal from the DHPR triggers SR Ca^{2+} release via RyR1, and a retrograde signal

from RyR1 enhances the L-type Ca^{2+} current via the DHPR. RyR1 (5,037 residues) assembles as a homotetramer in which the C-termini form the ion conducting pore across the SR membrane, with the remainder of the protein forming a ''foot'' structure which spans between the SR and plasma membranes. In an accompanying abstract (Polster et al., this meeting) we showed that a construct (YFP-$RyR1_{1:4300}$) which lacks the C-terminal residues was diffusely distributed in dysgenic myotubes (DHPR null), but that in dyspedic myotubes (RyR1-null but containing DHPRs) it targeted junctionally and retrogradely enhanced L-type Ca^{2+} current. Here we have examined the oligormerization of YFP-$RyR1_{1:4300}$. Measurement of FRAP for YFP-$RyR1_{1:4300}$ in dysgenic myotubes yielded a diffusion coefficient of $2.17x10^{-8}$ cm^2/sec, which, based on previous measurements of protein diffusion in muscle, is compatible with YFP-$RyR1_{1:4300}$ existing as a tetramer. We also used SDS PAGE (4-15% gradient) and immunoblotting to compare YFP-$RyR1_{1:4300}$ (expressed in tsA-201 cells) and native RyR1. Without prior cross-linking, YFP-$RyR1_{1:4300}$ displayed a smaller apparent MW than wt RyR1; after glutaraldehyde cross-linking, both proteins migrated as single bands of much higher apparent MW, with YFP-$RyR1_{1:4300}$ again displaying a slightly greater mobility, consistent with it's being a tetramer.

2284-Pos Board B303

The Cytoplasmic Domain of the RyR1 Foot is Sufficient for DHPR (Cav1.1) Organization into Tetrads

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A RyR1 construct that lacks the channel forming C-terminal residues but includes the entire cytoplasmic foot region (YFP-$RyR1_{1:4300}$) forms a stable tetrameric structure (H. Bichraoui et al., abstract this meeting), colocalizes with DHPRs at SR/plasmalemma junctions and retrogradely enhances peak DHPR currents when expressed in dyspedic (RyR1 null) myotubes (A. Polster et al., abstract this meeting). We tested the interaction of the expressed $RyR1_{1:4300}$ with DHPRs in dyspedic myotubes by examining the DHPR disposition using freeze-fracture. Normally DHPRs are targeted to junctional sites in the absence of RyR, but their organization into tetrads and the arrangement of tetrads in ordered arrays is strictly dependent on their link to arrays of tetrameric RyRs. Thus the disposition of DHPRs in freeze-fracture images provides direct information both on DHPR/RyR interaction and on the arrangement of RyRs. In dyspedic cells DHPR clusters at peripheral couplings in a loose, completely random arrangement. Cells expressing full-length RyR1 show more tightly clustered DHPRs and the presence of complete (4 elements) and incomplete (2-3 elements) tetrads that are aligned in an orthogonal array. Cells expressing the truncated $RyR1_{1:4300}$ show small well identifiable DHPR foci with closely spaced particles. Some but not all foci show clear grouping of DHPRs into complete tetrads and/or tetrads composed of only three elements, but the tetrads are not aligned into a orthogonal array. We conclude that similar to intact RyR1, $RyR1_{1:4300}$ forms homo-tetramers which can link to DHPRs and organize them into tetrads, but that the cytoplasmic RyR1 foot differs from full-length RyR1 in that it does not form orthogonal arrays. The related positioning of $RyR1_{1:4300}$ and DHPRs is necessary for the retrograde interaction between the two.

2285-Pos Board B304

Two Regions are Involved in Ca^{2+}-Dependent Inactivation of Ryanodine Receptor Calcium Channels

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Skeletal (RyR1) and cardiac muscle (RyR2) isoforms of ryanodine receptor calcium channels are ~65 % homologous in amino acid sequences; however they differ in their regulation by endogenous molecules and proteins. Both RyR1 and RyR2 are inhibited by millimollar Ca^{2+}, but RyR2 affinity for inhibitory Ca^{2+} is ~10 times lower than RyR1. Previous studies demonstrated that C-terminus quarter of RyR has critical domain(s) for Ca^{2+} inactivation (Du and MacLennan (1999) *J. Biol. Chem.*274, 26120; Nakai et al. (1999) *FEBS Lett.*459, 154). We pursued these observations to obtain further insights into RyR regulation by Ca^{2+}. We constructed and expressed 8 RyR1/RyR2 chimeras in HEK293 cells and determined Ca^{2+} activation and inactivation affinities of these channels by [3H]ryanodine ligand binding method. We found that replacing two regions of RyR1 with corresponding RyR2 sequence reduced the affinity for Ca^{2+} inactivation. The first region (RyR2 amino acid 4020-4250) contains two EF hand Ca^{2+} binding motifs (EF1: 4036-4047, EF2: 4071-4082). Another chimera containing only EF2 of RyR2 (RyR2 4053-4250) has only a modest (not significant) change in Ca^{2+} inactivation. The results suggest that EF1 is a critical determinant for RyR inactivation by Ca^{2+}. Consistently, a chimera channel carrying only RyR2-EF1 (RyR2 3692-4052) showed significantly reduced Ca^{2+} inactivation, whereas a chimera carrying RyR2 3692-4019 (no EF hands) behaved essentially as RyR1. Second, preliminary studies indicate that substitution of the last ~450 amino acids of RyR1 with corresponding RyR2 (4521-4968) results in Ca^{2+} inactivation affinity between RyR1 and RyR2. Replacing both regions in RyR1 with RyR2 sequences (4020-4250 and 4521-4968) further reduced Ca^{2+} inactivation affinity, which suggests that in addition to EF1, C-terminal ~450 amino acids have a role in Ca^{2+}-dependent inactivation of RyRs. Supported by NIH, AHA and NSF.

2286-Pos Board B305

FRET Detection of CaM-RyR2 Binding Modulation by S100A1

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Using fluorescence resonance energy transfer (FRET), we are testing the hypothesis that S100A1 competes with calmodulin (CaM) for binding to cardiac ryanodine receptors (RyR2). In isolated pig cardiac sarcoplasmic reticulum (SR) vesicles, we targeted a donor-labeled FKBP (D-FKBP) to the RyR2 cytosolic headpiece. We then detected FRET as a decrease of donor fluorescence in the presence of CaM labeled with acceptor within the N- or C-lobe (denoted A_N-CaM and A_C-CaM, respectively), thus directly and specifically indexing CaM binding in the proximity of D-FKBP. FRET between D-FKBP and A-CaMs (100 nM) was completely inhibited by unlabeled WT-CaM, with $K_I \approx 100$ nM, indicating that WT-CaM and A-CaMs compete with similar affinities for the same RyR2 binding site. However, S100A1 (ranging from 0.1 to 30 μM) had no significant effect on FRET when cardiac SR was concurrently incubated with S100A1 and A_N-CaM. Upon sequentially incubating the SR with S100A1 first, then with A_N-CaM, we found partial inhibition of FRET, but with $K_I \approx 30$ μM S100A1. This effect is more pronounced in nM Ca^{2+} (*versus* μM Ca^{2+}). Intriguingly, FRET between D-FKBP and A_C-CaM was not significantly affected by S100A1. S100A1 lowered maximum FRET for A_N-CaM but did not significantly change its binding affinity. This suggests that S100A1 allosterically interacts with RyR-CaM binding, rather than directly competing for the same binding site as CaM. We are currently developing a complementary FRET approach, using acceptor-labeled S100A1, to specifically resolve the S100A1 binding to RyR. Ultimately, we aim to elucidate the interplay between S100A1 and CaM binding to RyR.

2287-Pos Board B306

Benzodiazepines and Benzothiazepines as Modulators of the Sarcoplasmic Reticulum Calcium ATP-ase and Ryanodine Receptors in Striated Muscle

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We have reported that CGP-37157, a benzothiazepine (BZT) derivative of clonazepam utilized as a blocker of the mitochondrial Na^+/Ca^{2+} exchanger, also activates ryanodine receptors (RyRs) and inhibits the sarcoplasmic reticulum (SR) Ca^{2+}-stimulated ATPase (SERCA). We extended the studies to other BZT (e.g., clonazepan, diltiazem) as well as to benzodiazepines (BZD; e.g. diazepan, lorazepan). We aimed to determine if these drug classes have as a common trait the ability to modulate RyRs and/or SERCA. The effects of BZD and BZT on RyRs activity were tested in SR microsomes with a Ca^{2+} leak assay as well as after reconstituting RyRs into lipid bilayers. The agents tested had variable potency to increase RyR-mediated Ca^{2+} leak from skeletal SR microsomes. As an example, while diazepam significantly increased RyR activity, most of the others (clonazepam, lorazepam) had minor effects at high doses. In contrast, diltiazem produced moderate inhibition. Planar bilayer studies confirmed the leak observations with both cardiac and skeletal RyR. In the presence of ruthenium red, most agents decreased the rate of loading which indicates inhibitory effects on SERCA activity. The effects of BZT and BZD on loading correlated with a decrease in ATPase activity of SERCA-enriched skeletal SR fractions. In summary, most BZT and BZD utilized in therapeutics or as pharmacological tools have an inhibitory action on SERCA. In contrast, they show a variety of effects on RyRs ranging from inhibition to activation. Hence, the pharmacological action of BZT and BZD on cellular Ca^{2+} homeostasis reported in the literature of cardiac and skeletal muscle as well as other non-muscle systems may require taking into consideration the contributions of all drug-sensitive intracellular Ca^{2+} transporters. (Supported by NIH-GM078665 and AHA-MWA 12180038).